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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Withers et al.

Serial No.: 09/837,711 Examiner:E. Slobodyansky

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For : Methods and Compositions for the Synthesis of Oligosaccharides Using Mutant Glycosidase Enzymes

DECLARATION UNDER RULE 132

1. The undersigned, a named inventor of the above-captioned application, hereby submit the following declaration in support of the response to the Official Action mailed November 21, 2001. This declaration describes glycosynthase derived from *E. coli* LacZ β-galactosidase. The term "glycosynthase" has been adopted in the art to describe a mutant enzyme that has lost its normal glycolytic glycosidase function, while retaining the ability to catalyze a coupling reaction.

2. Wild-type LacZ β-galactosidase has within the active site a glutamic acid residue as amino acid 537 which is catalytically active. In the mutant form described in this declaration, this glutamic acid is replaced with a smaller amino acid with a non-carboxylic acid side chain, specifically serine. Coupling of β-D-glucopyranosides and an α-D-galactosyl fluoride, or β-D-cellobioside and an α-D-galactosyl fluoride are demonstrated. Thus, it is clear that the modifications to the active site of LacZ β-galactosidase as described in the present specification result in an enzyme which can be used in the method of claim 40. An even more effective galactosynthase was generated by the additional mutation of Gly794 to Asp.

3. **Enzyme mutagenesis, expression and purification**:

The plasmid pRSETlacZ was obtained from Invitrogen and codes for a N-terminal His-tagged lacZ gene under the control of a T7 promoter. It was mutated using the Promega

GeneEditor *in vitro* Site-Directed Mutagenesis System as described in the manual with the following oligonucleotide to generate the Glu537Ser mutation:
5'-GATCCTTTGCAGTTACGCCCACG-3'

(the mutated codon is underlined). The Gly794Asp mutation was introduced into the wild-type gene using the Promega kit with the following oligonucleotide
5'-AACGACATTGACGTCAGTGAAGCGACC-3'
Both mutants were digested with SacI and AccI restriction endonucleases and run on a 2 % agarose gel. The 5000 bp and 800 bp fragments were cut out and purified using a Qiaex II gel extraction kit. The 5000 bp vector backbone from mutantGlu537Ser was ligated with the 800 bp insert encoding Gly794Asp and transformed into XL1-Blue cells to afford the double mutant Glu537Ser Gly794Asp. Mutations were confirmed by sequence analysis.

The plasmid coding for Glu537Ser was transformed by electroporation into *E. coli* BL21 λDE3 and induced at $O.D._{600} = 0.6$ with IPTG (0.4 mM final concentration) and allowed to grow for a further four hours. Cells were harvested by centrifugation and passed twice through a French press at 4°C. The mutant, Glu537Ser, was isolated from the supernatant by Ni^{2+} chelation chromatography (His-bind resin, Novagen); yields of 50 mgL^{-1} were obtained. Protein was concentrated using Centricon Plus 20 kDa molecular weight cut off membranes.

$E_{280}^{0.1\%}$ Strain W2244 (*E. coli* genetic stock centre) was lysogenized with phage λDE3 using the Novagen λDE3 lysogenisation protocol. Plasmids coding for Glu537Ser and Glu537Ser Gly794Asp mutants were transformed into electrocompetent W2244 λDE3 cells. Protein expression and purification in W224 λDE3 was performed exactly as described for strain BL21 λDE3 except that final yields were 5 mgL^{-1}. Protein concentrations were determined by absorbance at 280 nm using the extinction coefficient = 2.1 cm^{-1}, determined from the amino acid sequence.

4. **Oligosaccharide synthesis and characterization:**

Enzyme reactions were performed in 200 mM phosphate buffer, pH 7.5, 1 mM $MgCl_2$. Reaction mixtures were monitored by TLC (Kieselgel 60 F_{254} (Merck), 7:2:1 ethyl acetate/methanol/water) and visualized under UV light and by exposure to 10 % ammonium molybdate in 2 M H_2SO_4. Reaction mixtures were passed through a 30 kDa ultrafiltration membrane to remove protein, applied to a TosaHaas Amide 80 HPLC column and separated by gradient elution of 80 % acetonitrile to 60 % acetonitrile. After analysis and identification by mass spectrometry (ESI), products were acetylated with acetic anhydride/pyridine (1:2) and purified by flash chromatography (ethyl acetate / hexanes) on silica gel (230 – 400 mesh). ^1H and ^{13}C NMR (Bruker Avance, 400 MHz) spectra were internally referenced to the solvent.

5. **4-Nitrophenyl β-D-galactopyranosyl-(1,6)-β-D-glucopyranoside:**

4-Nitrophenyl β-D-glucopyranoside (8.7 mg, 29.1 μM) and α-D-galactosyl fluoride (10.6 mg, 58 μM) were dissolved in phosphate buffer (1 mL) containing LacZ E537S (34 :M). After incubation at room temperature for 48 hours, with TLC monitoring, the reaction mixture was purified to yield the disaccharide (5.4 mg, 40 %). An identical reaction was performed for the double mutant, with the exception that LacZ E537S G794D (17 :M) was used as catalyst to yield the disaccharide (9.4 mg, 70 %).

6. **4-Nitrophenyl β-D-galactopyranosyl-(1,6)-β-D-glucopyranosyl-(1,4)-β-D-glucopyranoside**

4-Nitrophenyl β-cellobioside (10.6 mg, 22 μM) and α-D-galactosyl fluoride (18.4 mg, 100 μM) was dissolved in phosphate buffer (1 mL) containing LacZ E537S (17 :M). After incubation at room temperature for 48 hours, with TLC monitoring, the reaction mixture was purified to yield the trisaccharide (7.0 mg, 51 %). An identical reaction was performed for the double mutant, with the exception that LacZ E537S G794D (17 :M) was used as catalyst, yielding the trisaccharide (11.0 mg, 80 %).

7. **Phenyl β-D-galactopyranosyl-(1-6)-β-D-glucopyranoside**

Phenyl β-D-glucopyranoside (7.2 mg, 28 μM) and α-D-galactosyl fluoride (18.2 mg, 100 μM) were dissolved in phosphate buffer (1 mL) containing LacZ E537S (17 :M). After incubation at room temperature for 48 hours, with TLC monitoring, the reaction mixture was purified to yield the disaccharide (7.1 mg, 61 %). An identical reaction was performed for the double mutant, with the exception that LacZ E537S G794D (17 :M) was used as catalyst to yield the disaccharide (10.0 mg, 85 %).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

dated: 20th May 2002

Stephen G. Withers